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(54) Title: SELECTION-GENE-FREE TRANSGENIC PLANTS

(57) Abstract

Methods and materials for producing transgenic plants which are transgenic only with respect to foreign genes whose inclusion is desired, free of genes which confer selection characteristics. Advantage is taken of systems which mark DNA for excision in combination with enzymes mediating the excision to segregate selectable genes from desired transfer genes. In particular, the cre/lox system of bacteriophage P1 is used as an illustration of this method.

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SELECTION-GENE-FREE TRANSGENIC PLANTS

Technical Field

The invention is directed to materials and methods which produce transgenic plants containing only desired foreign genes and which are free of unwanted or irrelevant selection genes. Advantage is taken of recombinase systems which permit the excision and segregation of selection gene DNA from introduced genetic material.

Background and Related Art

Efforts to obtain transgenic plants with improved properties have been under way for some time. Transgenic plants have been obtained which are insect resistant, for example, due to the presence of a gene from Bacillus thuringiensis that confers such insect resistance. The nutritional value of various plants has also been improved by insertion of genes encoding proteins rich in desired amino acids. Introduction of genes encoding viral resistance mechanisms, herbicide resistance mechanisms, improved growth characteristics and the like are all desirable outcomes that can be achieved within the parameters of conventional technology in at least illustrative cases.

Included within the parameters of current technology is, however, the necessity of transferring, along with the desired DNA, DNA encoding a selection gene and means for its expression to permit the efficient recovery of successful transformants. Without these

selection genes, the successful transformants would be lost in the greatly dominant nontransformed population of plants or plant cells. The presence of the DNA encoding the selection genes and the products of these genes is not necessarily harmful, but does increase the environmental uncertainty of the distribution of transgenic plants. The problems introduced by the uncertainty of the ultimate effects of the inclusion and propagation of selection genes in plants has a highly negative effect on the progress of plant improvement. At a minimum, reservations expressed at various levels of intensity and clamor have political effects which retard the progress of obtaining improved plant species.

The present invention removes this source of environmental impact uncertainty by providing a method for excision and segregation of the selection gene which, after the initial transformation, has outlived its usefulness. The invention takes advantage of recombinase systems which are capable of excising marked DNA sequences.

One efficient system for excision of unwanted DNA sequences that has been widely employed generally is the Cre/lox system. This system, illustrative of the general process, comprises "lox" marker sequences that, when included in a DNA sequence per se, mark the DNA which they bracket for excision or inversion (depending on the orientation of the lox sequences) by a corresponding "Cre" recombinase enzyme. The operability of this system in various host cells, including tobacco cells, has been shown. For example, Sauer, Mol Cell Biol (1987) 7:2087-2096, showed the operability of the Cre/lox system in the yeast Saccharomyces cerevisiae. The operability of the system in mammalian cells was also shown by Sauer, B., et al., in Proc Natl Acad Sci USA

(1988) 85:5166-5170; Sauer, B., et al., Nucleic Acids Res (1989) 17:147-161. The operability of the Cre/lox system in tobacco cells was demonstrated by Dale, E., et al., in an abstract published in J Cell Biochem, Supplement 14E, "Abstracts of the 19th Annual UCLA Symposium for Molecular Strategies for Crop Improvement," page 1978 (16 April 1990), and in an article by Dale, E., et al., Gene (1990) 91:79-85.

The use of the Cre/lox system in yeast to regulate expression was also described by Sauer in European application 220,009, published 29 April 1987. Expression of a foreign gene during growth phase was prevented by strategies that precluded expression in the absence of the cre enzyme. The gene encoding the recombinase enzyme cre was placed under the control of an inducible promoter. The expression system for the desired gene was constructed either to be blocked by a DNA sequence marked by the lox sequences for excision, to be repressed by a repressor protein whose gene is marked by the lox sequences for excision, or to satisfy the condition in which the desired gene is designed to be contained in an inverted position in its expression system and bracketed by lox whereby the cre enzyme produced when induction takes place inverts the gene to the correct orientation. In this last strategy, the lox sequences bracket the desired sequence in opposite orientations.

Odell, J., et al., in Mol Gen Genet (1990) 223:369-378, describe the use of the Cre/lox system in higher plants to activate the expression of the kanamycin resistance gene into the chromosome. The authors further suggest the use of the system to regulate expression in higher plants in a manner similar to that described in the above-referenced EPO application for yeast.

While the Cre/lox system has been demonstrated to be particularly useful, other such recombinase systems exist and are derived from a variety of sources. For example, O'Gorman, S., et al., in Science (1991) 251:1351-1355, describe the FLP recombinase system from S. cerevisiae as operable in mammalian cells; Golic, K.G., et al., Cell (1989) 59:499-509, report the operability of this system in Drosophila. Backman, K.C., U.S. Patent 4,673,640, describes a marker/recombinase system from bacteriophage lambda, and Matsuzaki, H., et al., J Bacteriol (1990) 172:610-618 describe an analogous system from the yeast C. rouxii.

Thus, it appears that recombinase/marker systems are available from a variety of sources and are functional in a number of hosts. The present invention specifically takes advantage of such recombinase/marker systems to excise and segregate selection genes in higher plants.

Disclosure of the Invention

The invention includes a means to control the nature of transgenic plants so as to provide plants which contain only the desired transgenic material and lack the selection genes useful in conducting the transformations necessary for their preparation. The resulting plants thus have more predictable environmental effects than those previously available in the art. The invention methods take advantage of recombinase/marker systems for excision and segregation of selection marker genes.

Thus, in one aspect, the invention is directed to vectors suitable for transformation of higher plants which comprise selection genes marked by DNA marker sequences which form a part of a recombinase/marker system. The selection gene is further linked to control

sequences which are capable of effecting its expression so that it can be used in the selection of transformed plants prior to excision. The invention also includes these vectors that further contain an expression system for a desired gene. The invention is also directed to plant cells or cultures in regenerated plants transformed with the vectors of the invention and to plant cells and plants which are further transformed with expression vectors that include the gene for the recombinase enzyme associated with the recombinase marker system. The invention is also directed to methods to produce such transformants and to segregate the selection gene from the desired gene in progeny.

Brief Description of the Drawings

Figure 1 is a schematic showing the construction of pED37 and pED53. As indicated in Figure 1, the pED37 oriented as shown will be inserted into the SalI site of pED53 in the recombined vector pED53::pED37.

Figure 2A shows the positioning of the insert from pED53::pED37 into the host genome and the expected sizes of PCR products obtained from primers as described in the examples hereinbelow.

Figure 2B shows the expected orientation of this insert in the gene after excision of the hpt selection gene. The expected PCR product size is also shown.

Figure 3 shows the sequence of the amplified portions indicated in Figures 2A and 2B.

Modes of Carrying Out the Invention

The invention is directed to methods which result in transgenic plants that are capable of expressing a desired gene but that are free of genes

associated with the transformation process as "selectable markers." The invention methods provide, therefore, plants having conferred characteristics which are limited to those desired to be achieved by the transformation and not associated with unrelated, and perhaps unwanted selection characteristics.

In general, these plants are obtained by either of two related methods. Both methods require that the selection gene associated with the initial transformation with the desired gene be operably linked to marker DNA sequences that mark the gene for excision mediated by a corresponding recombinase enzyme. In both methods, therefore, plants or plant cells are initially transformed with a recombinant vector which contains a selection characteristic gene operably linked to marker DNA sequences that mark the gene for excision, the marked gene being contained in an expression system so that unless excised, the gene is expressed. The vector further includes an expression system which comprises the desired gene operably linked to appropriate control sequences. Of course, the expression systems must be operable in higher plant cells. Initial transformants are selected by virtue of the presence of the selection gene contained in the recombinant vector and can be verified to express, as well, the gene for the desired characteristic.

In one approach, initially transformed plant cells or plants can be further transformed in a second round of transformation with a second recombinant vector which contains the recombinase gene that corresponds to the DNA sequences that mark the selection gene for excision. In order to verify the second transformation, of course, a second selection gene needs to be included so that the second round of transformation can be

verified. The successful second transformants, for the most part, will be free of the first selection marker due to the operation of the recombinase product of the recombinase gene. Among those transformants free of the first selection marker, progeny are screened for freedom from the second selection marker as well. As the second selection marker is not linked to the desired gene construct, it is segregated from the desired gene in the progeny.

Typically, this first approach is conducted by transformation of plant cells, and regenerating the screened progeny into intact plants. Alternatively, the second transformants can first be regenerated into plants and self-pollinated to produce the segregated progeny.

In a second approach, regenerated plants from the first and second transformations (which are conducted independently) are cross-pollinated to effect the excision of the first selection marker. The progeny of the products of this cross-pollination that show expression of the desired gene can then be screened for the absence of the second selection gene. As the desired gene and the second selection gene are not linked, segregation occurs in these second generation progeny.

As used herein, a "selection characteristic gene" or "selection gene" refers to a gene which produces a product that confers on a host containing it a selectable property such as herbicide or antibiotic resistance. Suitable selection genes for methods of the invention include genes encoding hygromycin resistance (the hpt gene) or kanamycin resistance (nptII gene) and any other gene which confers such characteristics on higher plant cells.

As used herein, a "desired gene" is a gene that may encode a protein or an RNA, the presence of which is

desired in the finished plant. Such genes may include those encoding seed storage proteins, those encoding insect resistance, or any other gene which is thought to confer desired characteristics on the plant containing it.

As used herein, "expression system" has its usual meaning--i.e., DNA which contains the coding sequence of a gene operably linked to control systems which effect the expression of the gene in the higher plant cells. Typically, such control sequences include a promoter, and optionally, additional sequences which aid in expression such as polyadenylation sites, or other appropriate control sequences.

"Marker DNA sequences" refer to sequences that, when present in proper orientation with respect to included DNA, mark the gene for excision or inversion when in the presence of an appropriate corresponding enzyme. "Corresponding recombinase" refers to the enzyme which is capable of recognizing the marker DNA sequences and excising or inverting the included DNA. Illustrated herein is the system that includes the lox DNA sequences corresponding to the cre recombinase enzyme, but a multiplicity of such systems are known. These are reviewed, for example, by Craig, Annual Review of Genetics (1988) 22:77-105. Any such system operable in higher plants may be used.

In constructing the vectors containing expression systems useful in the invention, control regions which are functional either constitutively are employed. Transcription initiation regions, for example, include the various opine initiation regions, such as octopine, mannopine, nopaline and the like. Plant viral promoters can also be used, such as the cauliflower mosaic virus 35S promoter.

A large number of suitable control systems are available. For example, the cauliflower mosaic virus (CaMV) 35S promoter has been shown to be highly active in many plant organs and during many stages of development when integrated into the genome of transgenic plants including tobacco and petunia, and has been shown to confer expression in protoplasts of both dicots and monocots.

Organ-specific promoters are also well known, but may be less convenient. For example, the E8 promoter is only transcriptionally activated during tomato fruit ripening, and can be used to target gene expression in ripening tomato fruit (Deikman and Fischer, EMBO J (1988) 7:3315; Giovannoni et al., The Plant Cell (1989) 1:53). The activity of the E8 promoter is not limited to tomato fruit, but is thought to be compatible with any system wherein ethylene activates biological processes.

Either a constitutive promoter (such as the CaMV or Nos promoter illustrated above) or a desired organ-specific promoter (such as the E8 promoter from tomato) is then ligated to the gene to be expressed using standard techniques now common in the art. The expression system may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements.

Thus, for expression in plants, the recombinant expression cassette will contain in addition to the coding sequence, a plant promoter region, a transcription initiation site (if the coding sequence to be transcribed lacks one), and a transcription termination sequence. Unique restriction enzyme sites at the 5′ and 3′ ends of the cassette may also be included to allow for easy insertion into a pre-existing vector.

Sequences controlling eucaryotic gene expression have been extensively studied. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. By convention, the start site is called +1. Sequences extending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G(or T)NG (Messing, J. et al., in Genetic Engineering in Plants, Kosage, Meredith and Hollaender, eds. (1983) pp. 221-227). Other sequences conferring tissue specificity, response to environmental signals, or maximum efficiency of transcription may also be found in the promoter region. Such sequences are often found within 400 bp of the transcription initiation site, but may extend as far as 2000 bp or more.

In the construction of heterologous promoter/structural gene combinations, the promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

As stated above, any of a number of promoters which direct transcription in plant cells is suitable. The promoter can be either constitutive or inducible. Promoters of bacterial origin include the octopine

synthase promoter, the nopaline synthase promoter and other promoters derived from native Ti plasmids (Herrera-Estrella et al., Nature (1983) 303:209-213). Viral promoters include the 35S and 19S RNA promoters of cauliflower mosaic virus (O'Dell et al., Nature (1985) 313:810-812). Plant promoters include the ribulose-1,3-disphosphate carboxylase small subunit promoter and the phaseolin promoter.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

If the mRNA encoded by the structural gene is to be efficiently processed, DNA sequences which direct polyadenylation of the RNA are also commonly added to the vector construct (Alber and Kawasaki, Mol and Appl Genet, (1982) 1:419-434). Polyadenylation is of importance for expression of the transcription product RNA in plant cells. Polyadenylation sequences include, but are not limited to the Agrobacterium octopine synthase signal (Gielen et al., EMBO J, (1984) 3:835-846) or the nopaline synthase signal (Depicker et al., Mol and Appl Genet (1982) 1:561-573).

The resulting expression system or cassette is ligated into or otherwise constructed to be included in a recombinant vector which is appropriate for higher plant transformation.

As is particularly relevant herein, the vector will also contain an expression system for a selection gene by which transformed plant cells can be identified in culture. Usually, the selection gene will encode antibiotic resistance, e.g., resistance to G418,

hygromycin, bleomycin, kanamycin, and gentamicin. After transforming the plant cells, those cells having the vector will be identified by their ability to grow on a medium containing the particular antibiotic. Replication sequences, of bacterial or viral origin, are generally also included to allow the vector to be cloned in a bacterial or phage host, preferably a broad host range procaryotic origin of replication is included. A selection gene suitable for bacteria should also be included to allow selection of bacterial cells bearing the desired construct. Suitable procaryotic selection genes also include resistance to antibiotics such as kanamycin or tetracycline.

Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art. For instance, in the case of Agrobacterium transformations, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

In addition, vectors can also be constructed that contain in-frame ligations between the coding sequence of the desired gene and sequences encoding other molecules of interest resulting in fusion proteins, by techniques well known in the art.

When an appropriate vector is obtained, transgenic plants are prepared which contain the desired expression system. A number of techniques are available for transformation of plants or plant cells. All types of plants are appropriate subjects for "direct" transformation; in general, only dicots can be transformed using Agrobacterium-mediated infection.

In one form of direct transformation, the vector is microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA (Crossway, Mol Gen Genetics (1985) 202:179-185). In

another form, the genetic material is transferred into the plant cell using polyethylene glycol (Krens, et al., Nature (1982) 296:72-74), or high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, is used (Klein, et al., Nature (1987) 327:70-73). In still another method protoplasts are fused with other entities which contain the DNA whose introduction is desired. These entities are minicells, cells, lysosomes or other fusible lipid-surfaced bodies (Fraley, et al., Proc Natl Acad Sci USA (1982) 79:1859-1863).

DNA may also be introduced into the plant cells by electroporation (Fromm et al., Proc Natl Acad Sci USA (1985) 82:5824). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

For transformation mediated by bacterial infection, a plant cell is infected with Agrobacterium tumefaciens or A. rhizogenes previously transformed with the DNA to be introduced. Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (A. tumefaciens) and hairy root disease (A. rhizogenes). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the

presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of A. tumefaciens or the Ri plasmid of A. rhizogenes. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome (Schell, J., Science (1987) 237:1176-1183). Ti and Ri plasmids contain two regions essential for the production of transformed cells. One of these, named transferred DNA (T-DNA), is transferred to plant nuclei and induces tumor or root formation. The other, termed the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. The T-DNA will be transferred into a plant cell even if the vir region is on a different plasmid (Hoekema, et al., Nature (1983) 303:179-189). The transferred DNA region can be increased in size by the insertion of heterologous DNA without its ability to be transferred being affected. Thus a modified Ti or Ri plasmid, in which the disease-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell.

Construction of recombinant Ti and Ri plasmids in general follows methods typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to "shuttle vectors," (Ruvkum and Ausubel, Nature (1981) 298:85-88), promoters (Lawton et al., Plant Mol Biol (1987) 9:315-324) and structural genes for antibiotic

resistance as a selection factor (Fraley et al., Proc Natl Acad Sci (1983) 80:4803-4807).

There are two classes of recombinant Ti and Ri plasmid vector systems now in use. In one class, called "cointegrate," the shuttle vector containing the gene of interest is inserted by genetic recombination into a non-oncogenic Ti plasmid that contains both the cis-acting and trans-acting elements required for plant transformation as, for example, in the pMLJ1 shuttle vector of DeBlock et al., EMBO J (1984) 3:1681-1689 and the non-oncogenic Ti plasmid pGV3850 described by Zambryski et al., EMBO J (1983) 2:2143-2150. In the second class or "binary" system, the gene of interest is inserted into a shuttle vector containing the cis-acting elements required for plant transformation. The other necessary functions are provided in trans by the non-oncogenic Ti plasmid as exemplified by the pBIN19 shuttle vector described by Bevan, Nucleic Acids Research (1984) 12:8711-8721 and the non-oncogenic Ti plasmid PAL4404 described by Hoekema, et al., Nature (1983) 303:179-180. Some of these vectors are commercially available.

There are two common ways to transform plant cells with Agrobacterium: co-cultivation of Agrobacterium with cultured isolated protoplasts, or transformation of intact cells or tissues with Agrobacterium. The first requires an established culture system that allows for culturing protoplasts and subsequent plant regeneration from cultured protoplasts. The second method requires (a) that the intact plant tissues, such as cotyledons, can be transformed by Agrobacterium and (b) that the transformed cells or tissues can be induced to regenerate into whole plants.

Most dicot species can be transformed by Agrobacterium as all species which ar a natural plant

host for Agrobacterium are transformable in vitro. Monocotyledonous plants, and in particular, cereals, are not natural hosts to Agrobacterium. Attempts to transform them using Agrobacterium have been unsuccessful until recently (Hooykas-Van Slogteren et al., Nature (1984) 311:763-764). However, there is growing evidence now that certain monocots can be transformed by Agrobacterium. Using novel experimental approaches cereal species such as rye (de la Pena et al., Nature (1987) 325:274-276), maize (Rhodes et al., Science (1988) 240:204-207), and rice (Shimamoto et al., Nature (1989) 338:274-276) may now be transformed.

Identification of transformed cells or plants is generally accomplished by including a selectable marker in the transforming vector, or by obtaining evidence of successful bacterial infection.

Plant cells which have been transformed can also be regenerated using known techniques.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. II, 1986). It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beet, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently root d. Alternatively, somatic embryo

formation can be induced in the callus tissue. These somatic embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and plant hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable. After the expression cassette is stably incorporated into regenerated transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Construction of Plasmids

Plasmid pED23 which contains the expression system for the cre gene was constructed as described by Dale, E.D. and Ow, D.W., Gene (1990) 91:79-85. This plasmid uses a pUC19 host vector (Yanich-Perron, C. et al. Gene (1985) 33:103-119) and utilizes a 35S promoter to transcribe a Cre-nos3' fusion.

The plasmid pED53 is constructed from the Agrobacterium gene transfer vector pBIN19, the construction of which was described by Bevan, M., Nucleic Acids Res (1984) 12:8711-8721. To convert pBIN19 to pED53, a 1.3 kb PstI fragment was deleted. The deletion removes part of the T-DNA including the coding region of the kanamycin resistance gene (nptII) along with HindIII and SphI sites.

Plasmid pED37 was constructed from pED26, which contains the luciferase expression system and has been described by Dale, E.C. and Ow, D.W. (supra) by inserting a 2.0 kb HindIII fragment containing the 35S-hpt-nos3' chimeric gene. The hpt gene is described by Kaster, K.R. et al. Nucleic Acids Res (1983) 11:6895-6911.

The co-integrate plasmid pED53::pED37 was formed by linearizing pED37 at an XhoI site adjacent to one of the lox sites and inserting it into the SalI site of pED53. As shown in Figure 1, pED37 contains the 35S promoter operably linked to the luciferase gene which is in turn terminated by the nos3' sequence. Bracketed by the lox sites are ampicillin resistance, and the hpt expression system. The pED37 is co-integrated into pED53 between the right and left border (RB and LB) regions in the orientation shown.

The co-integrate plasmid which supplies the cre gene, pBIN19::pED23 was obtained by ligating the HindIII linearized pED23 into the HindIII site of pBIN19 so that the transcription of the cre gene is directed toward the Agrobacterium LB.

The co-integrate plasmids were selected by ability to confer resistance to both kanamycin and ampicillin in bacteria. This selection is facilitated by transformation into the E. coli polymerase I-deficient host JZ294 (argH, strA, polA::Tn10) which permits replication of the wide host range replicons of pED53 and pBIN19 but not the ColE1 replicons of pED37 and pED23.

Example 2

Production of Selectable Marker-Free Plants

The co-integrate plasmids pED53::pED37 and pBIN19::pED23 were mobilized into Agrobacterium tumefaciens strain GV3111(pTiB6S3SE) for infection of

Nicotiana tabacum (Wisconsin-38 cultivar) leaf explants as described by Horsch, R.B. et al. Science (1985) 227:229-231. Hyg^R and Kan^R plants were scored for the ability of leaf explants to form shoots on shoot-inducing MS media containing antibiotics (20 μg/ml hygromycin sulfate or 100 μg/ml kanamycin sulfate). Luc^+ plants were assayed for luciferase activity as described by Ow, D.W. et al., Science (1986) 34:856-859.

Initially, hygromycin-resistant plants were obtained from transformation with pED53::pED37. These are designated "ntED5337" plants. These plants contain the luciferase expression system and the hpt expression system incorporated into the genome in the configuration shown in Figure 2A. This was verified by genetic analysis as described below. The ntED5337 plants express the luciferase gene, and are hygromycin-resistant as required by the selection protocol.

The ntED5337 plants were transfected, using the protocol described above, with pED23. Successful transformants will be selectable for kanamycin resistance and are capable of production of the cre enzyme. The resulting transformants were thus selected by kanamycin resistance and the kanamycin-resistant plants were then tested for hygromycin resistance as described above. Most of them, designated ntED5337-23, were hygromycin-sensitive. This showed that the introduction of the cre enzyme catalyzed recombination of the lox sites flanking the hygromycin resistance gene. The deleted DNA, no longer linked to the replicating host chromosome, is lost in the progeny cells deriving from the primary cell where the excision event occurred. The ntED5337-23 plant cells or the regenerated plants express the gene encoding luciferase, however.

In addition to the protocol described above, plants regenerated from independent transformations by pED23::pED37 and pBIN19::pED23, respectively, were cross-pollinated to obtain progeny. Of 316 progeny examined, 78 produced luciferase and were kanamycin-resistant. Among these, 42 were hygromycin-sensitive. These 42 plants are substantially equivalent to those regenerated from the cellular progeny ntED5337-23.

Example 3

Genetic Analysis

To verify the genetic status of the transformants, DNA was prepared by grinding leaf tissues in liquid N_2, extracting with 100 mM Tris/1% SDS/50 mM EDTA/500 mM NaCl/10 mM β-mercaptoethanol at 65°C, 10 min., followed by adding potassium acetate to 1.3 M, chilling to 0°C and removing the debris by centrifugation. The DNA was then precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 mM Tris/1.0 mM EDTA. Polymerase chain reactions were carried out under standard conditions as described by Saiki, R.K. et al., Science (1988) 239:487-491 with denaturation, annealing, and extension at 94°C, 55°C and 72°C, respectively for 1 min. each during 30 cycles. Reaction products were resolved using a 1.5% agarose gel. The sequence of the PCR primers in Figures 2A and 2B are: A, 5'-GAGCTCGGTACCCGGGGATC-3'; B, 5'-GAGTGCACCATATGCGGTGT-3'; C, 5'-GACGCCCCAGCACTCGTCCG-3'; D, 5'-GGTACCCGGGATCCTCTAG-3'; E, 5'-GTTCATTTCATTTGGAGAGG-3'; F, 5'-CAGTGATACACATGGGGATC-3'. The two primers for detection of the cre gene (5'-ATGTCCAATTTACTGACCGT-3' and 5'-CTAATCGCCATCTTCCAGCA-3') represent the N and C-terminal cre coding sequence and the expected PCR product size is 1.0 kb. To det rmine the sequence of the lox

sites, fragments from PCR reactions were purified, digested with appropriate restriction enzymes (A+B; BamHI/NdeI, C+D; PstI/BamHI, A+D; ClaI/BamHI) and ligated into either pUC19 (for A+B and C+D) or pBR322 (for A+D). The nucleotide sequences of the regions surrounding the lox sites derived form PCR reactions from plants ntED5337 and ntED5337-23 were determined by the dideoxy method as modified by U.S. Biochemical (Sequenase kit).

Five of the hygromycin-sensitive, kanamycin-resistant and luciferase-producing plants derived from the secondary transformation described in Example 2 were analyzed to confirm site-specific recombination at the lox sites. Both parental ntED5337 and derivative ntED5337-23 plants were examined. The predicted resultants are shown in Figure 2A and 2B. As confirmed by gel electrophoresis, a fragment of the predicted size of 1.1 kb for primers A+B and 0.71 kb for primers C+D was obtained from the parent genome, but not from ntED5337-23. However, primer A+C produced a 0.87 kb band from ntED5337-23, but not from the parent. This band corresponds to the fragment expected from the joining of the chimeric luciferase gene with the sequence adjacent to the LB. Primers E+F were used to assay for the possibility that the hygromycin resistance gene might have translocated elsewhere in the genome; a fragment corresponding to the expected 0.56 kb band was found in the parent, but not in the descendent ntED5337-23. Thus, the excised hygromycin gene is not present in the genome at all. The excision event also removes the silent ampicillin resistance marker from the plant genome.

Of five plants examined, the PCR profiles showed no indication of harboring both excised and intact copies as shown in Figure 2A and 2B suggesting that the

excision must have occurred early between introduction of the cre-expressing construct and plant organogenesis.

For sequencing plant ntED5337 derived fragments produced from PCR amplification by primers A+B and primers C+D, as well as the ntED5337-23 derived fragment from primers A+D were cloned into plasmid vectors. Figure 3 shows the experimentally obtained sequences of the three different lox-containing regions. The lox regions derived form primers A+B and C+D share 80 bp of identity that include the 34 kb lox sequence, but diverge outside of this segment. As shown in Figure 3, each parental lox sequence is adjacent to one characteristic restriction site, either SalI or BamHI, but on opposite sides of the lox sequence. The lox sequence of the PCR product derived from primers A+D from ntED5337-23 DNA, however, is flanked by both restriction sites and by sequences found in opposing sides of each of the parental ntED5337 lox sites. As in bacteria, the recombination event within plant chromatin was conservative, i.e., without loss or alteration of the lox sequence or its flanking DNA. To our knowledge, this is the first description at the nucleotide sequence level of cre/lox-catalyzed recombination in eucaryotic chromosomal DNA.

Example 4
Segregation

Two ntED5337-23 plants were self-pollinated to allow segregation of the luciferase gene from the cre locus which also harbors the linked nptII selection gene. Approximately 100 R_1 germinated seedlings from each self-pollinated plant were scored for luciferase activity. In both cases, about 3/4 of the total progeny produced luciferase and among these, approximately 1/4 were expected to be sensitive to kanamycin. This was the case

with the progeny from one plant. From the second plant, however, only 1 out of approximately every luciferase producer progeny was sensitive to kanamycin. This indicated either genetic linkage between the cre-nptII and the luc loci or that there were two unlinked copies of the cre-nptII construct. It is conceivable that more than a single gene transfer event occurred during the second round of Agrobacterium infection. PCR analysis of DNA prepared from luciferase-producing seedlings using primers internal to the cre gene sequence also showed independent segregation of the cre-nptII locus at the same time frequencies obtained in the screen for the kanamycin sensitive phenotype. The loss of the cre-nptII locus, as determined by the PCR analysis, was confirmed phenotypically by an inability of leaf explants to form shoots in the presence of kanamycin. A more extensive PCR analysis of the luc locus (as described above) of three representative $Luc^{+}Hyg^{S}Kan^{S}$ R_1 plants showed that they have the same profile of fragments as described for the R_0 ntED5337-23 plants. Hence, in two generations (R_0 and R_1), we have shown the feasibility of transferring a gene into the plant genome without incorporating a selectable marker.

CLAIMS

1. A recombinant vector operable in higher plants which vector comprises:

a selection characteristic gene operably linked to marker DNA sequences that mark said gene for excision mediated by a corresponding recombinase enzyme, said selection gene further operably linked to control sequences capable of effecting the expression of said selection gene in a higher plant.

2. The vector of claim 1 which further includes an expression system operable in higher plants to express a desired gene which expression system comprises said desired gene operably linked to control sequences capable of effecting the expression of said desired gene in a higher plant.

3. The vector of claim 2 wherein said desired gene and said selection gene are operably linked to the same control sequences.

4. The vector of claim 2 wherein said desired gene and said selection gene are operably linked to different control sequences.

5. A higher plant cell or plant cell culture transformed with the vector of claim 2.

6. A regenerated transgenic higher plant transformed with the vector of claim 2.

7. The plant cell or plant cell culture of claim 5 which is further transformed with a s cond vector

operable in higher plants which vector comprises an expression system for a gene encoding a recombinase protein corresponding to said marker DNA sequences, which expression system comprises said recombinase encoding gene operably linked to control sequences capable of effecting the expression of said recombinase gene in a higher plant.

8. The regenerated transgenic plant of claim 6 which is further transformed with a second vector operable in higher plants which vector comprises an expression system for a gene encoding a recombinase protein corresponding to said marker DNA sequences, which expression system comprises said recombinase encoding gene operably linked to control sequences capable of effecting the expression of said recombinase gene in a higher plant.

9. A method to obtain a plant cell or plant cell culture suitable for regeneration to a transgenic plant, which method comprises transforming the plant cell or plant cell culture of claim 5 with a second vector which comprises an expression system for a recombinase protein corresponding to said marker DNA sequences, which expression system comprises said recombinase encoding gene operably linked to control sequences capable of effecting the expression of said recombinase gene in a higher plant.

10. The method of claim 9 wherein said second vector further includes a second selection gene operably linked to control sequences capable of effecting the expression of said second selection gene in a higher plant.

11. The method of claim 10 which further includes regenerating said plant cells to obtain a regenerated higher plant.

12. A method to obtain a regenerated higher plant transgenic with respect to a desired gene, which method comprises regenerating the plant cells of claim 10.

13. A method to obtain a transgenic plant containing only one or more desired foreign genes and free of selection genes, which method comprises:

(1) transforming plant cells or intact plants or portions thereof with a vector comprising a selection gene operably linked to marker DNA sequences which mark said selectable gene for excision and an expression system for said desired gene;

(2) selecting successful transformants exhibiting the characteristics of the gene product of the selection gene;

(3) further transforming said successful transformants with a second vector comprising an expression system for a recombinase protein corresponding to said marker sequences which mediates excision of said first selection gene and which further comprises an expression system for a second selection gene;

(4) selecting successful transformants from said further transforming step by selecting for the characteristics conferred by the gene product of said second selection gene;

(5) self-pollinating said successful transformants to obtain progeny therefrom; and

(6) screening said progeny for individuals that contain desired genes and lack said first and second selection genes.

14. A transgenic plant prepared by the method of claim 13.

15. A method to obtain a transgenic plant containing only one or more desired foreign genes and free of selection genes, which method comprises screening progeny resulting from self-pollination of transgenic plants which have been obtained by

(1) transforming plant cells or intact plants or portions thereof with a vector comprising a selection gene operably linked to marker DNA sequences which mark said selectable gene for excision and an expression system for said desired gene;

(2) selecting successful transformants exhibiting the characteristics of the gene product of the selection gene;

(3) further transforming said successful transformants with a second vector comprising an expression system for a recombinase protein corresponding to said marker sequences which mediates excision of said first selection gene and which further comprises an expression system for a second selection gene; and

(4) selecting successful transformants from said further transforming step by selecting for the characteristics conferred by the gene product of said second selection gene.

16. A transgenic plant prepared by the method of claim 15.

17. A method to obtain a transgenic plant containing only one or more desired foreign genes and free of selection genes, which method comprises screening progeny resulting from plant progeny which plant progeny have been obtained by cross-pollinating transgenic plants which have been obtained by:

(1) transforming plant cells or intact plants or portions thereof from a first plant in the cross with a vector comprising a first selection gene operably linked to marker DNA sequences which mark said selection gene for excision in an expression system for said desired gene;

(2) selecting successful transformants exhibiting the characteristics of the gene product of the selection gene;

(3) transforming plant cells or intact plants or portions thereof of a second plant in the cross with a vector comprising an expression system for a recombinase protein corresponding to said marker DNA sequence which mediates excision of said first selection gene and which further comprises an expression system for a second selection gene;

(4) screening plant progeny from the cross-pollination for the characteristics conferred by the gene product of said second selection gene and do not express the first selection gene;

(5) self-pollinating said progeny to obtain progeny therefrom; and

(6) screening said progeny for individuals that contain desired genes and lack said first and second selection genes.

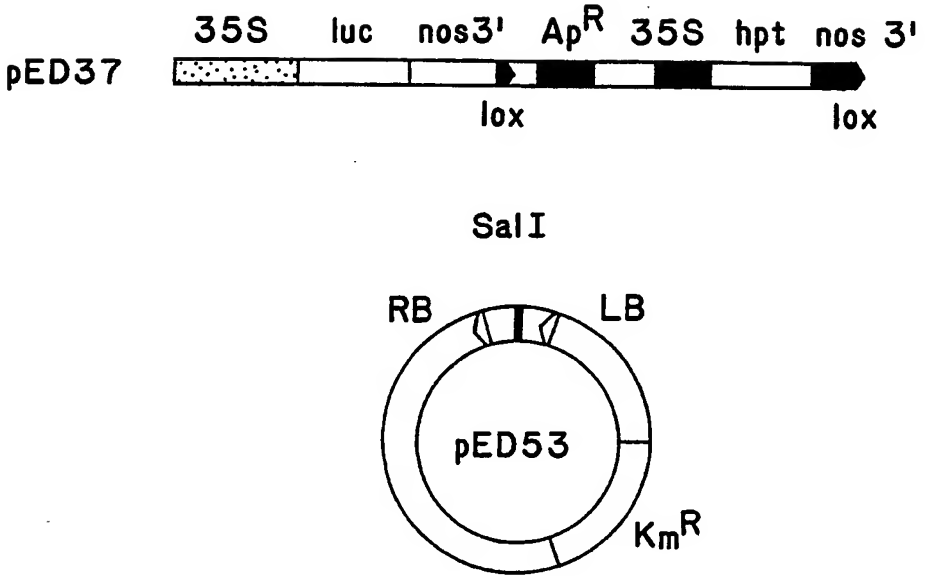
35S
luc
nos3'
Ap^R
35S
hpt
nos 3'
pED37
lox
lox
SalI
RB
LB
pED53
Km^R

FIG. 1

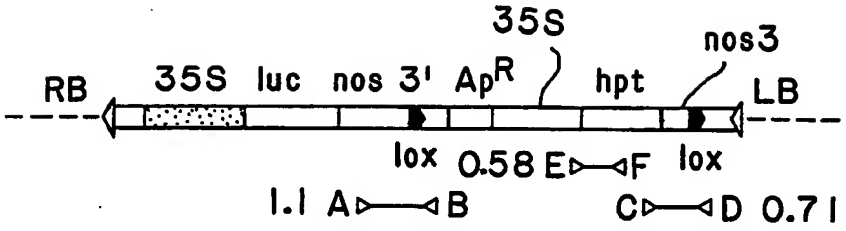
35S
nos3
RB
35S luc nos 3' Ap^R
hpt
LB
lox
0.58 E
F
lox
1.1 A
B
C
D 0.71

FIG. 2A

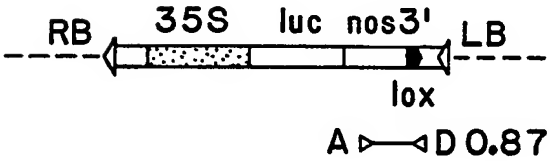
RB
35S luc nos3'
LB
lox
A
D 0.87

FIG. 2B


ATAACTTCGTATAATGTATGCTATACGAACTTAT
Sal I
(A+B) luc-nos -TCACAAATAGCCC TCGACTCTAGAGGATCGATCCGGAACCCTTAAT- lox -TAGGTCCCTCGAC CCGAGCTCGAAT- pUC19
(A+D)
G
A
T
C
lox
TCTAGAGGATCC
(C+D) hpt-nos -AAGCTTCGAGGGCC TCGACTCTAGAGGATCGATCCGGAACCCTTAAT- lox -TAGGTCCCTCGAC TCTAGAGGATCC- pED53
BamHI

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05640

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 5/00; A01H 1/00, 1/04
US CL :800/205; 435/172.3, 240.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.1, 172.3, 240.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,959,317 (Sauer, B L.) 25 September 1990, col. 8, lines 31-68, col.s 9-16, lines 1-68, col. 17, lines 1-14.	1-17
Y	Molecular and Cellular Biology, Volume 7, No. 6, issued June 1987, B. Sauer, "Functional Expression of the cre-lox Site-Specific Recombination System in the Yeast Saccharomyces cerevisiae", pages 2087-2096, especially pages 2091-2094.	1-17
Y	Nucleic Acids Research, Volume 17, No. 1, issued 1989, B. Sauer and N. Henderson, "Cre-Stimulated Recombination at loxP-Containing DNA Sequences Placed Into The Mammalian Genome", pages 147-161, especially pages 156-158.	1-17
Y	Bio/Technology, Volume 7, No. 12, issued December 1989, P. Knight, "Engineered Fruit and Vegetable Crops", pages 1233-1237, especially page 1235	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
07 SEPTEMBER 1992	17 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHARLES RORIES Telephone No. (703) 308-1120

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